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Effects of CO_2 enrichment on two microalgae species: A toxicity approach using consecutive generations

Esther Bautista-Chamizo [a, *], Ana R. Borrero-Santiago [b], Manoela R. De Orte [c, 1], Ángel DelValls [a, 2], Inmaculada Riba [a]

[a] Departamento de Química-Física, Facultad de Ciencias del Mar y Ambientales, Universidad de Cádiz, Puerto Real, Cádiz, Spain
[b] Department of Chemistry, Norwegian University of Science and Technology, Trondheim, Norway
[c] Departamento de Ciencias do Mar, Universidade Federal de São Paulo, Santos, SP, Brazil

HIGHLIGHTS

- The paper addresses the potential impacts of CO_2 enrichment in the marine environment.
- Two different marine microalgae species were used through four consecutive generations.
- *T. chuii* showed a slight adaptation through generations, in terms of metabolic activity.
- *P. tricornutum* was the most sensitive one with almost total growth inhibition in the fourth generation.
- The results give valuable data about the transgenerational effects of CO_2 enrichment on microalgae.

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ABSTRACT

As a result of the increasing pressure provoked by anthropogenic activities, the world climate is changing and oceans health is in danger. One of the most important factors affecting the marine environment is the well-known process called ocean acidification. Also, there are other natural or anthropogenic processes that produce an enrichment of CO_2 in the marine environment (CO_2 leakages from Carbon Capture and Storage technologies (CCS), organic matter diagenesis, volcanic vents, etc). Most of the studies related to acidification of the marine environment by enrichment of CO_2 have been focused on short-term experiments. To evaluate the effects related to CO_2 enrichment, laboratory-scale experiments were performed using the marine microalgae *Tetraselmis chuii* and *Phaeodactylum tricornutum*. Three different pH values (two treatments - pH 7.4 and 6.0 - and a control - pH 8.0) were tested on the selected species across four consecutive generations. Seawater was collected and exposed to different scenarios of CO_2 enrichment by means of CO_2 injection. The results showed different effects depending on the species and the generation used. Effects on *T. chuii* were shown on cell density, chlorophyll-*a* and metabolic activity, however, a slight adaptation across generations was found in this last parameter. *P. tricornutum* was more sensitive to acidification conditions through generations, with practically total growth inhibition in the fourth one. The conclusions obtained in this work are useful to address the potential ecological risk related to acidification by enrichment of CO_2 on the marine ecosystem by using consecutive generations of microalgae.

1. Introduction

Oceans health is under high and still increasing pressure from the current climate change provoked by anthropogenic activities (Wernberg et al., 2011). Anthropogenic carbon emissions are mainly originated by the burning of fossil fuel and worsened because of deforestation (Bijma et al., 2013). CO_2 values have increased from 280 ppm in the preindustrial era (IPCC, 2007) until

* Corresponding author.
E-mail addresses: esther.bautista@uca.es (E. Bautista-Chamizo), ana.r.borrero@ntnu.no (A.R. Borrero-Santiago), manoela.romano@gmail.com (M.R. De Orte), angel.valls@uca.es (Á. DelValls), inmaculada.riba@uca.es (I. Riba).
[1] Present address: Department of Global Ecology, Carnegie Institution, Stanford, California, USA.
[2] Alternative address: Departamento de Ecotoxicologia, Universidade de Santa Cecília, Santos, SP, Brazil.

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the current 411.25 ppm measured at Mauna Loa (NOAA, May 2018).

While renewable energy sources seem to be the key to mitigating climate change, the Carbon Capture and Storage (CCS) technology has been suggested as the best temporarily option to reduce carbon emissions (EPA, 2014). But this technique is associated with a great concern about the risk that a potential CO_2 leakage could cause in the marine environment (Ardelan et al., 2009). Therefore, the CCS technology is considered another potential source of acidification by CO_2 enrichment (Khosrovyan et al., 2014; Pasarelli et al., 2017; De Orte et al., 2018).

The immediate effects of climate change on oceans are warming (IPCC, 2007) and acidification, since oceans act as a sink of CO_2 (Sabine et al., 2004). There is a continuous flux of CO_2 across the interface atmosphere-ocean, which causes the alteration of the seawater chemistry, with the consequent phenomena known as ocean acidification (OA) (Raven, 2005). Previous studies related to this issue point out that although some marine species will be tolerant to these changes, the whole ecosystem balance could be damaged (Turley and Gatusso, 2012).

There are also many natural sources of CO_2 enrichment in the marine environment, such as the organic matter degradation and diagenesis in the sediment (Canfield, 1994), the natural CO_2 vents (Hall-Spencer et al., 2008; McGinnis et al., 2011) or the submarine eruption in the Canary Islands (Spain) where pH values between 5.13 and 8.04 have been measured (Santana-Casiano et al., 2012).

As it has been suggested in multiple research works, marine acidification will cause a wide range of responses from individuals but also from ecosystems as a whole (Riebesell et al., 2000; Zondervan, 2007). Many contradictory trends have been reported about those effects of acidification on marine ecosystems; including positive, neutral or negative responses, depending on the physiological processes involved. The magnitude of acidification effects on microalgae has been proved to be a species-specific issue (Hinga, 2002). In the case of microbial communities, the processes affected by acidification include primary productivity, trace gases emission, nitrogen fixation and organic matter degradation, among others (Das and Mangwani, 2015). Changes on the ecosystem are related to the potential modification of its composition, structure, energy flow and resources (Blackford, 2010) with still unknown consequences to the environment.

Microalgae play an essential role on ecosystems (Cairns et al., 1992). Being the base of the marine trophic chain, higher trophic levels depend on this group (McCormick and Cairns, 1994) which means that any change in these organisms could provoke severe consequences in the marine ecosystem. Ocean acidification research has been focused on studies with photoautotrophic organisms such as phytoplankton, since photosynthesis is a key process in elemental cycles, giving energy to higher trophic levels by the organic matter production, using CO_2 and inorganic nutrients (Riebesell et al., 2010). Some attributes of microalgae including short life cycle, high sensibility to environmental stressors and rapid growth, give them the role of a productive indicator on climate change (McCormick and Cairns, 1994). Also it is remarkable to mention that microorganisms' assays are not subject to ethical restrictions, different from investigation with higher organisms such as fish or invertebrates (Debelius et al., 2009).

The majority of research in this topic is focused on short-term studies (Kroeker et al., 2013). For this reason, the knowledge on multi-generational effects of acidification in marine ecosystems is limited (Reusch, 2014; Sunday et al., 2014). This lack of knowledge is limiting the comprehension of how marine ecosystems will deal with acidification (Rodriguez-Romero et al., 2015). Therefore, there is a risk to over or under estimate the species responses to this phenomenon.

The aim of this study is to evaluate the effects of acidification by CO_2 enrichment in two marine microalgae species across consecutive generations, using the species *Tetraselmis chuii* and *Phaeodactylum tricornutum*. Acidification scenarios were conducted in laboratory with a CO_2 injection system. Bubbling CO_2 into the water is a very efficient technique to manipulate carbonate chemistry and it is one of the five best practices recommended by Riebesell et al. (2010). The election of the pH values was based on two potential phenomena. On the one hand, the process known as anthropogenic ocean acidification caused by the increase of CO_2 concentration in the atmosphere derived from human activities (pH 7.4). This pH has been established according to the predictions of a decrease in the pH of 0.5 units for 2100 (Caldeira and Wickett, 2003). On the other hand, the pH 6.0 scenario based on a potential CO_2 leakage from sub-seabed carbon capture and storage (CCS) emplacement (Pasarelli et al., 2017; De Orte et al., 2018). Also, another scenario was tested as a control (pH 8.0). The species chosen for this work are equally relevant for the ecosystems and research: *Tetraselmis chuii,* which has been deeply studied for its adequacy on biodiesel production and as a CO_2 sink, and *Phaeodactylum tricornutum*, a widely used species on toxicity tests because of its sensitiveness. Both of them are important species in aquaculture as a nutrient for multiple species. This work pretends to raise awareness about the potential risks of the pH decrease in seawater to the marine ecosystem as a whole, working under the hypothesis that a scenario of acidification by CO_2 enrichment may represent a danger for the development of microalgae not only immediately but also across generations.

2. Material and methods

2.1. The CO_2 Injection System

The CO_2 Injection System® (P201200753, Cádiz) has been developed for the simulation of the CO_2 enrichment process in laboratory, through the use of toxicity bioassays (Fig. 1). With this system, organisms are exposed to different pH values in order to assess the potential adverse effects of acidification by enrichment of CO_2 on marine ecosystem. This experiment was developed according to the methodology described in Bautista-Chamizo et al. (2016).

2.2. Seawater analysis

Seawater aliquots (50 mL of filtered sample) were analysed with an automatic alkalinity titrator (Metler Toledo, T50). Carbonate system speciation was calculated using the measured values of pH and total alkalinity (TA), with the CO2SYS software (Pierrot et al., 2006) with constant dissociation from Mehrbach et al. (1973) refit by Dickson and Millero (1987) and KSO_4 according to Dickson (1990).

2.3. Toxicity tests

The microalgae species (*Tetraselmis chuii* and *Phaeodactylum tricornutum*) were obtained from “Servicios Centrales de Investigación en Cultivos Marinos” at the University of Cádiz. Experiments were performed in triplicate using Erlenmeyer flasks previously sterilized through HNO_3 (10%) washes and autoclaved. Temperature was kept at 22 °C (±2) and illumination was provided by eight fluorescent lights (36 W) in continuous. Each flask was filled with 200 mL of filtered (Millipore 0.22 μm) seawater (pH 8.0 ± 0.1, salinity 34) enriched with f/2 medium (Sunda and Guillard, 1976). Exponentially-growing populations of microalgae were exposed to three acidification scenarios defined by different pH values (8.0 as a control, 7.4 and 6.0). The initial cell density was

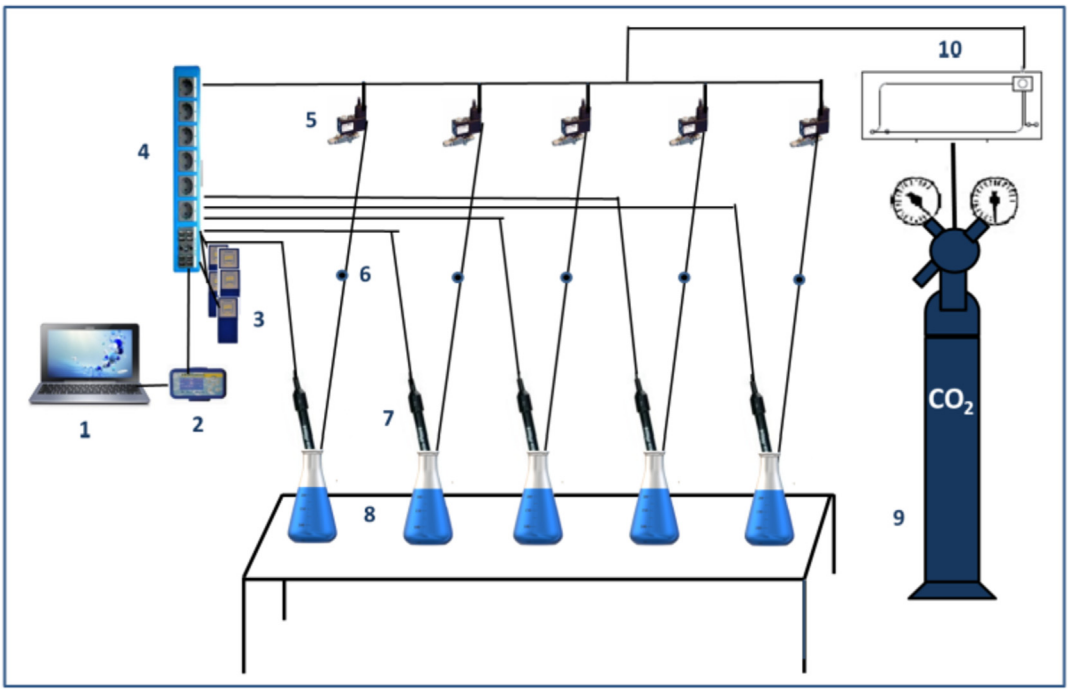

Fig. 1. The CO_2 Injection System®. 1. Laptop with software (Aquamedic 8.0). 2. AT-Control System. 3. pH interfaces to connect the pH sensor to the AT-Control System. 4. Power strips and USB connectors. 5. Solenoid valves for the electronic regulation of the CO_2 injection. 6. Retrovalves. 7. pH sensor. 8. Erlenmeyer flask. 9. CO_2 bottles. 10. Semi-automatic station of CO_2 gas supply.

established at 5×10^5 cells mL^{-1} for *Tetraselmis chuii* and 10^6 cells mL^{-1} for *Phaeodactylum tricornutum*. The experiments were performed during four consecutive generations, for a total of 16 days. All the parameters were measured at the end of each generation (every 96 h). Toxicity tests were developed according to international standards (OECD, 1984).

2.3.1. Cell density

To calculate the microalgae cell density after 96 h, 5 mL aliquot were collected and fixed with Lugol. Cell density was determined with a Neubauer chamber in an optical Microscope (Olympus CKX41).

2.3.2. Photosynthetic pigment content

Samples (20 mL) were filtered using glass microfiber filters (GF/C Whatman). Pigments were extracted in a 90% acetone solution (10 mL), in darkness and refrigeration (4 °C). After that, 3 mL of the centrifuged extracting solution were taken for the spectrophotometer analysis at determined wavelengths (480, 630, 647, 664, and 750 nm). Data were calculated using the trichromatic equation of Jeffrey and Humphrey (1975) for chlorophyll-*a* (Chl-*a*):

1) Chl-*a*: $[\text{Chl-}a] = 11.85 * A_{664} \rightarrow 1.54 * A_{647} \rightarrow 0.08 * A_{630}$

2.3.3. Metabolic activity

Metabolic activity was determined spectrophotometrically with a cell esterase activity assay using fluorescein diacetate (FDA). The suitability of this technique as a method to detect alterations in the metabolic activity of phytoplankton as a consequence of environmental changes or pollution had previously been proved (Jochem, 1999). FDA is a non-polar and non-fluorescent molecule, which is able to cross the cell membranes freely. Inside the cell, non-specific esterases fragment the FDA molecule into a fluorescing fluorescein and two acetates. Due to its polarity, the fluorescein is captured into the cells, showing cell membrane integrity and the amount of fluorescence will increase according to the metabolic activity of those mentioned esterases (Prado et al., 2009). To develop this methodology, two samples of each replicate were stained (from now on "sample *a*" (T_0) and "sample *b*" (T_1)) with a FDA stock solution (on a final concentration of 10 $\mu L\, mL^{-1}$). Immediately, acetone in a proportion 1:1 was added to sample *a* to stop the reaction and was kept in the fridge (4 °C), while sample b was incubated under same laboratory conditions as the experiment. After 3 h, acetone was added to sample *b*. All the samples were then centrifuged at 4500 rpm for 20 min and 4 °C. After that, 3 mL of supernatant were extracted and absorbance was read in the spectrophotometer (490 nm). Metabolic activity results were obtained from T_1-T_0 absorbance. A calibration curve was developed with several concentration of fluorescein, using acetone/water (proportion 1:1) as a blank.

2.4. Statistical analysis

Data were analysed using the statistical software SPSS 15.0 for Windows. Variation in growth, metabolic activity and chlorophyll-*a* among the different pH values and generations for each species were studied with an analysis of variance (two-way ANOVA). Confidence intervals (95%) were calculated in order to evaluate the significant differences ($p < 0.05$) among the pH treatments compared to the pH control in each generation and species for all the studied parameters.

3. Results

3.1. Seawater analysis

Table 1 includes the data for carbonate system speciation calculated at all pH treatments. The carbon parameters were calculated based on initial values of pH (8.0, 7.4 and 6.0),

Table 1
Carbonate system speciation in seawater for each pH (pH 8.0-control, pH 7.4 and pH 6.0), temperature (22 °C) and salinity (34). Abbreviations: total alkalinity (TA), total inorganic carbon (TIC), bicarbonate ion concentration (HCO_3^-), carbonate ion concentration (CO_3^{2-}), carbon dioxide concentration (CO_2), partial pressure of carbon dioxide (pCO_2), calcite saturation state (Ω_{Cal}) and aragonite saturation state (Ω_{Arag}).

Parameter	pH 8.0	pH 7.4	pH 6.0
TA (μmol/kg SW)	2585.1	2479.3	2347.3
TIC (μmol/kg SW)	2388.0	2710.6	4739.5
HCO_3^- (μmol/kg SW)	2215.7	2296.8	2350.1
CO_3^{2-} (μmol/kg SW)	154.2	21.2	1.3
CO_2 (μmol/kg SW)	22.5	216.2	2386.2
pCO_2 (μatm)	729.8	6941.2	77799.0
Ω_{Cal}	3.76	0.61	0.04
Ω_{Arag}	2.35	0.45	0.03

temperature (22 °C), salinity (34), and TA. Results showed that total inorganic carbon (TIC) was higher as pH was reduced, while carbonate ion concentration (CO_3^{2-}) was reduced with the pH decrease. On the other hand, saturation index for calcite (Ω_{Cal}) and aragonite (Ω_{Arag}) decreased as pH decreased and pCO_2 increased with lower pH levels.

3.2. Microalgae response

3.2.1. Cell density

Fig. 2 (a) illustrates the cell density after 96 h for the microalgae *Tetraselmis chuii* exposed to three different pH values, two treatments (7.4, 6.0) and a control (non-acidified natural seawater, pH 8.0) across four generations. Significant increases ($p < 0.05$) among pH 7.4 and 6.0 compared with the control (pH 8.0) were observed in all generations. Fig. 2 (b) shows the cell density of *Phaeodactylum tricornutum* after 96 h of exposure to two different pH values (7.4 and 6.0) and non-acidified control (pH 8.0), across four generations. Significant differences were found in pH 7.4 (G2, G3 and G4) and pH 6.0 (G3 and G4) compared to the control of each generation (pH 8.0). In this species, a high decrease in cell density was found in G3 and G4 for pH 6.0. In both figures, cell density is expressed as a percentage, and data were normalized to the results obtained in the control (considered as 100% of cell density). Two-way ANOVA showed that the two factors individually and together (pH and generation) were significantly ($p < 0.05$) affecting cell density in both species (Table 2).

3.2.2. Metabolic activity

After 96 h of exposure, the esterase activity analysis revealed that the cell metabolic activity was significantly affected ($p < 0.05$) on both pH treatments and species, and for all the consecutive generations (except pH 7.4, G3, *T. chuii*), being highly reduced in the case of *P. tricornutum* (Fig. 3). In both figures, metabolic activity is expressed as a percentage, and data were normalized to the results obtained in the control (considered as 100% metabolically active). According to the two-way ANOVA results, the two factors (pH and generation) and all their possible combinations (except generation by itself in the case of *T. chuii*) were significantly ($p < 0.05$) interfering in metabolic activity.

3.2.3. Photosynthetic pigment content

The concentration of chlorophyll-*a* per cell after 96 h for both species across four generations is represented in Fig. 4. Statistical analysis showed significant differences ($p < 0.05$) on *Tetraselmis chuii* between the control (pH 8.0) and the treatments (pH 7.4 and pH 6.0) for all generations. The pattern for this species showed an increase in chlorophyll-*a* with respect to the control. This increase was diminishing across generations. *Phaeodactylum tricornutum* showed significant differences between the control (pH 8.0) and the treatments pH 7.4 and 6.0 (G1, G3 and G4) and just pH 7.4 in G2. In both figures, chlorophyll-*a* is expressed as a percentage, and data was normalized to the control (considered as 100% of chlorophyll-*a*). Two-way ANOVA showed significant effects in chlorophyll-*a* due to the two factors and also due to their dual combination in both species.

4. Discussion

The increase of CO_2 in the marine environment is causing an unequivocally impact in seawater chemistry (Sabine et al., 2004). CO_2 dissolved in the ocean reacts with seawater to form carbonic acid (H_2CO_3), which is dissociated to bicarbonate (HCO_3^-), carbonate (CO_3^-) and protons (H^+). When there is an excess of CO_2 the equilibrium of the carbonate speciation system is altered, modifying the buffer capacity of the seawater (Borrero-Santiago et al., 2017) which provokes a decrease of the pH values (Rost et al., 2008). However, increased CO_2 enrichment may be beneficial for marine phytoplankton because of the low affinity of their carboxylating enzyme (Rubisco) for CO_2 (Badger et al., 1998). Thus, enhanced phytoplankton growth and photosynthetic carbon fixation may occur in acidified environments (Riebesell et al., 1993; Riebesell and Gatusso, 2015).

Experiments based on long-term exposures to acidification have found controversial effects, with stimulation (Low-Décarie et al., 2013), reduction (Tatters et al., 2013) or neutral effects on growth

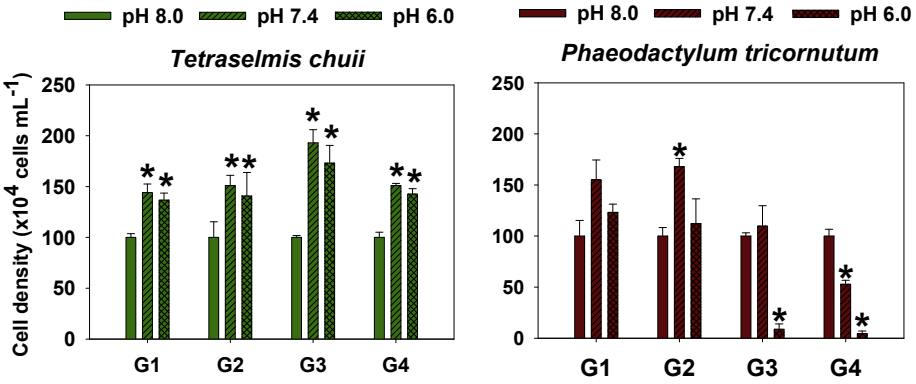

Fig. 2. a) *Tetraselmis chuii* cell density. Data represent the mean value for each pH and generation, including standard deviations, n = 3. b) *Phaeodactylum tricornutum* cell density. Data represent the mean value for each pH and generation, including standard deviations, n = 3. Significant differences are calculated compared to the control of each generation (pH 8.0) (*$p < 0.05$).

Table 2
Summary results of two-way ANOVA. Showing the f ratios and *p* values for the interactive effects of pH, species and generation on cell density, chlorophyll-*a*, and metabolic activity. Significant effects ($p < 0.05$) are indicated in bold.

Species	Parameter	pH	Generation (G)	pH × G
Tetraselmis chuii	Cell density	F = 175.057 $p = 0.000$	F = 59.899 $p = 0.000$	F = 9.651 $p = 0.000$
	Chlorophyll-*a*	F = 381.687 $p = 0.000$	F = 153.871 $p = 0.000$	F = 18.828 $p = 0.000$
	Metabolic activity	F = 9.435 $p = 0.001$	F = 1.598 $p = 0.216$	F = 3.851 $p = 0.008$
Phaeodactylum tricornutum	Cell density	F = 63.904 $p = 0.000$	F = 360.226 $p = 0.000$	F = 50.970 $p = 0.000$
	Chlorophyll-*a*	F = 72.127 $p = 0.000$	F = 146.727 $p = 0.000$	F = 93.819 $p = 0.000$
	Metabolic activity	F = 1178.016 $p = 0.000$	F = 18.509 $p = 0.000$	F = 4.016 $p = 0.006$

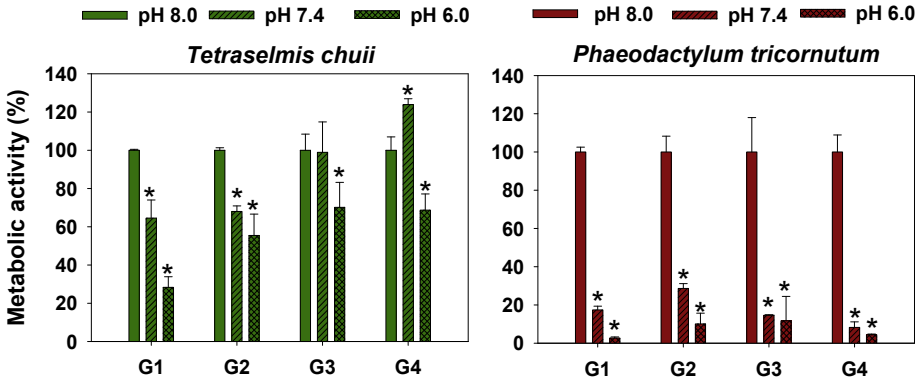

Fig. 3. a) *Tetraselmis chuii* metabolic activity. Data represent the value for each pH, for each generation, including standard deviations, n = 3. b) *Phaeodactylum tricornutum* metabolic activity. Data represent the mean value for each pH, for each generation, including standard deviations, n = 3. Significant differences are calculated compared to the control (pH 8.0) of each generation and represented by * ($p < 0.05$).

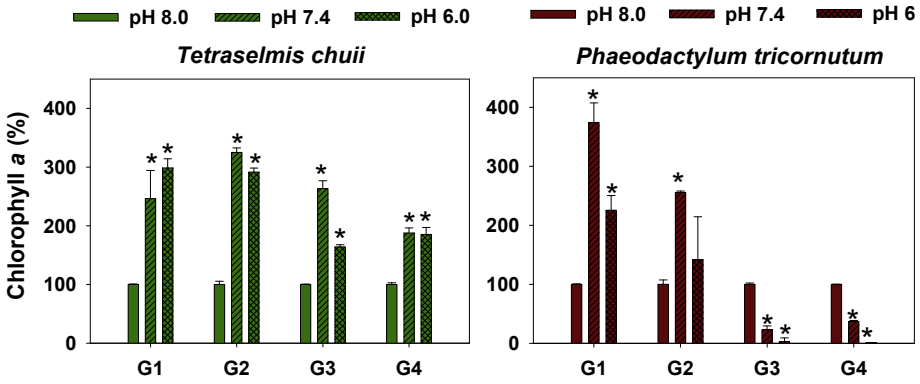

Fig. 4. a) *Tetraselmis chuii* chlorophyll-*a*. b) *Phaeodactylum tricornutum* chlorophyll-*a*. Data represent the value for each pH, for each generation, including standard deviations, n = 3. Significant differences are calculated compared to the control for each generation (pH 8.0) and represented by * ($p < 0.05$).

rates (Crawfurd et al., 2011). Also, an evolution of microalgal populations has been detected during long-term experiments (Jin et al., 2013; Schaum and Collins, 2014). In the present study, *T. chuii* population showed positive effects on growth during the four generations tested, with the stimulation of cell division, under pH 7.4 and pH 6.0. However, in the case of *P. tricornutum* neutral effects were shown until the third generation. From this generation onward, the cultures exposed to pH 6.0 were critically reduced. On the work developed by Li et al. (2017), *P. tricornutum* was tested in short and long-term exposure to pH ~ 7.7, showing two different strategies to acidification. On the one hand, shorter exposures showed plastic response to low pH, while longer exposure suggest a potential evolutionary strategy against high CO_2 concentrations. Our results are in accordance with those recorded in *M. aeruginosa* which experimented growth inhibition when exposed during 11 days to pH 6.2 (with four CO_2 aeration series per day) but it grew exponentially when aeration was programmed twice a day with a final pH of 7.01 (Wang et al., 2011).

Microalgae metabolic activity has been usually studied through photosynthesis, respiration and ATP synthesis (Gilbert et al., 1992; Greene et al., 1994). However, an alternative technique is based on the FDA metabolic probe which is absorbed by cells and metabolised by esterases (Gala and Giesy, 1994). In these experiments a decrease pattern of metabolic activity in *T. chuii* cultures exposed to low pH values (7.4 and 6.0) were shown. This behaviour was similar in both pH treatments, although it was more intense when exposed to pH 6.0. Thus, although this species growth was enhanced with CO_2 enrichment, metabolic activity was slowed down, as a response to acidification conditions (low pH values). However, it is important to remark that a potential adaptation to this acidification is observable, since the diminishing of metabolic activity was lower across generations for this species. In the case of *P. tricornutum* the decrease in metabolic activity was in accordance with the decrease in cell density, and apparently there was not recuperation across

generations. These results are in agreement with previous work where a decrease of metabolic activity was also detected on microalgae exposed to external environmental stressors such as the freshwater microalgae *M. aeruginosa* and *S. capricornutum* exposed to acid polluted waters (Regel et al., 2002). Decreases in fluorescein fluorescence have been interpreted as inhibition of esterase activity but may be also related to possible changes in membrane permeability and integrity (Franklin et al., 2000). In the study developed by Le Faucheur et al. (2011) the FDA hydrolysis by *C. reinhardtii* was used to determine the effect of acidification/pH on algal membrane permeability to lipophilic solutes. Also, FDA has been used as a biomarker for cell viability (Prado et al., 2012). Thus, these results may suggest that cells were rapidly dividing themselves as a response to high concentrations of CO_2. However, some of these cells may not be viable as shown in FDA results, which could explain why *P. tricornutum* was hardly growing under pH 6.0 in the third and fourth generations.

Chlorophyll-*a* concentration was increased in all generations for *T. chuii* exposed to low pH values. In the case of *P. tricornutum* this increase was recorded in the first two generations, while a decrease was shown in the third and fourth generation, which is in accordance with cell density results. However, this chlorophyll-*a* increase is not necessary a good symptom, since metabolic activity already showed toxic effects on cells. Thus, the increase in chlorophyll-*a* may suggest a decrease in the photosynthetic efficiency of cells, which is related to an obstruction in the electron transport chain, with the inhibition of the PSII reaction centre in the acceptor side (Murthy et al., 1990; Cid et al., 1995). Similar effects were found in Bautista-Chamizo et al. (2018) when microalgae cultures were exposed to the combination of low pH and high values of temperature and salinity.

It has been demonstrated that acidification has different effects on marine ecosystems, while some organisms will be enhanced with the CO_2 increase, others will be endangered. In addition, responses will vary depending on the species, with potential winners and losers (Hinga, 2002). But these responses will also change according to the type of acidification, while a progressive acidification can be faced with an adaptation of the organisms (Sunday et al., 2011; Pistevos et al., 2011), a sudden change in the seawater pH provoked by a CO_2 leakage would not give enough time for the ecosystem acclimatization (Basallote et al., 2012). Species specific response to changes in seawater acidification by CO_2 enrichment will have consequences on the phytoplankton natural species competition (Falkowsky and Oliver, 2007) but also on the evolution of phytoplankton taxa (Collins and Bell, 2004). Long-term effects in phytoplankton abundance and community structure as a consequence of seawater acidification may have many ecological implications in marine ecosystems. Microalgae are essential in global primary production (Field et al., 1998) which maintains the diversity and abundance of marine life and the ecosystem equilibrium (Nagelkerken and Connell, 2015). Hence, the alteration in phytoplankton production and species succession will have an impact on several marine biogeochemical cycles (Riebesell, 2004). Besides, any change in abundance or composition of the phytoplankton community will be transmitted to higher trophic levels, in particular to mesozooplankton population (Denoyelles et al., 1982; Rossoll et al., 2012) and therefore to commercial fisheries (Hays et al., 2005). Also, the microalgae population weakening may allow the alien species invasion (Hall-Spencer et al., 2008).

Thus, the CO_2 enrichment provoked by different sources like those mimicked in this study, specially the one related to a potential CO_2 leakage in the aquatic ecosystem, may have devastating effects on the phytoplankton community and therefore, in the food web.

5. Conclusions

The results from this study evidenced different sensitivity to the CO_2 enrichment between the species and across generations. While *T. chuii* was most resistant to acidification, showing an increase in cell density with low pH (pH 7.4 and pH 6.0) and a slight recuperation to acidification conditions in the fourth generation (in terms of metabolic activity), *P. tricornutum* cultures showed more drastic effects on metabolic activity (in all generations) and practically total growth inhibition in the fourth generation.

According to the results obtained in the present study and from the complementary research in the area to date, slow or progressive acidification processes such as those tested in this study (i.e. the future scenarios projected for ocean acidification processes, pH 7.4 or acidification from natural processes of CO_2 enrichment) may allow microalgae to develop adaptation strategies in terms of growth, photosynthesis and metabolic activity. However, this plasticity will not be as feasible if the pH drop occurs sharply in a short period time (i.e a CO_2 leakage from CCS technologies; pH 6.0). Thus the exposure period and the magnitude and intensity of the acidification process will be key in the effects provoked on the microalgae population, since these effects will be transmitted across generations. Although short-term laboratory experiments have given valuable information about the potential effects of acidification in phytoplankton communities to date, future studies in this topic should include multigenerational studies in order to obtain more realistic results about global changes impact in aquatic ecosystems. Hence, the results obtained from this work give valuable information about the transgenerational effects that CO_2 enrichment (related to low values of pH) may cause on microalgae species and set preliminary results as the basis for future experiments based on microalgae plasticity and evolution.

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